5-2014

Dissociation of the Effects of Serotonin Reuptake Inhibitor Fluoxetine in Prelimbic Cortex on Disruption of Timing and Working Memory for Time by Neutral and Negative Emotional Events

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by

Chance Christensen

Thesis submitted in partial fulfillment
of the requirements for the degree

of

DEPARTMENTAL HONORS

in

Human Movement Science in the Department of Health, Physical Education & Recreation

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UTAH STATE UNIVERSITY
Logan, UT
Spring 2014

Dissociation of the Effects of Serotonin Reuptake Inhibitor Fluoxetine in Prelimbic Cortex on Disruption of Timing and Working Memory for Time by Neutral and Negative Emotional Events

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Highest degree obtained: ARM, B.S.; CDC, B.S.; BZY, B.A.; MB, Ph.D., M.D.; CVB, Ph.D.

Running Title: Fluoxetine effect on interval timing with distracters

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Abstract

Learning and memory abilities are altered in disorders of the serotonergic system, in disorders such as such as depression, phobias, and post-traumatic stress disorder (PTSD). Among the processes impaired by emotional distracters, and whose dysregulation is documented in affective disorders, is the ability to time in the seconds-to-minutes range, i.e., interval timing. Presentation of distracters during timing tasks result in delays in responding suggesting a failure to maintain subjective time in working memory, as proposed by the Relative Time-Sharing (RTS) model. We investigated the role of the prelimbic cortex in the detrimental effect of anxiety-inducing distracters on the cognitive ability to interval time, using local infusions of selective serotonin reuptake inhibitor (SSRI) fluoxetine in a modified peak-interval procedure with neutral and anxiety-inducing distracters. Given that fluoxetine has beneficial effects on decreasing emotional responses to negative events, we hypothesized that fluoxetine would improve working memory in trials with distracters. Our results revealed a dissociation of the effects of fluoxetine infusion in the prelimbic cortex on interval timing and resource allocation (working memory for time), when neutral and anxiety-inducing distractions occurred. Fluoxetine was effective only during trials with distracters, but not during trials without distracters. Moreover, fluoxetine reduced the harmful effect of the distracters not only when the distracters were anxiety-inducing, but in fact exacerbated their detrimental effects on working memory when they were neutral. Results are discussed in relation to the brain circuits involved in RTS of resources, and the pharmacological management of affective disorders.

Keywords: Interval timing, serotonin, SSRI, fluoxetine, mPFC

Introduction

Learning, memory and emotional processing are dysregulated in affective disorders such as depression, phobias, and post-traumatic stress disorder (PTSD) resulting in anxiety and altered perception of events e.g. (Etkin *et al*, 2011; Gil and Droit-Volet, 2009; Hermann *et al*, 2009; Lui *et al*, 2011; Ritchey *et al*, 2011). For example, perception is hindered in individuals with PTSD (Terr, 1983), due to an attentional bias towards novel or emotionally charged stimuli (Kimble *et al*, 2000). Effective therapies for these disorders include traditional psychotherapy (Bleiberg and Markowitz, 2005), putative cognitive enhancers; (Steckler and Risbrough, 2012), and pharmacotherapy (Fletcher *et al*, 2010), prominently targeting serotonin. Indeed, among the most commonly prescribed antidepressants, 10 out of the top 12 target serotonin (Cipriani *et al*, 2009). In particular, selective serotonin reuptake inhibitors (SSRI) and putative cognitive enhancers (Floresco and Jentsch, 2011), such as fluoxetine (Prozac©), have beneficial reductive effects on affective disorder symptoms (Koen and Stein, 2011).

One locus of putative serotonergic action is the frontal cortex, responsible for higher-order executive tasks and functions (Robbins and Arnsten, 2009). Frontal cortex is strongly innervated by serotonin neurons (Lambe *et al*, 2011) and its damage has severe effects on personality and decision making (Van Horn *et al*, 2012). Given the prominent expression of serotonergic receptors in the prefrontal cortex (PFC), a strong modulatory effect by serotonin was ascribed to this region (Puig and Gulledge, 2011). Therefore, we sought to assess the effects of PFC fluoxetine infusion on cognitive function in the presence of neutral of negative emotional distracters.

Among the processes impaired by emotional distracters, and whose dysregulation is documented in affective disorders, is the ability to time in the seconds-to-minutes range, i.e., interval timing. Timekeeping is indeed affected in depression (Droit-Volet, 2013), phobias (Hermann *et al*, 2009), schizoaffective disorders (Lee *et al*, 2009) and PTSD (Terr, 1983). Presentation of task-irrelevant

distracters during a timing task results in a considerable delay in responding (Buhusi, 2012; Buhusi and Meck, 2009; Matthews *et al*, 2012), suggesting a failure to maintain subjective time in working memory, possibly due to attentional and working memory resources being diverted away from timing (Buhusi *et al*, 2009). Resource re-allocation is exacerbated by anxiety-inducing task-irrelevant distracters, resulting in impairing effects. Most interestingly for the purposes of this paper, antidepressant medication reduces the effect of emotional distracters on interval timing (Matthews *et al*, 2012).

Previous interval timing tasks have shown that the mPFC of is implicated in the ability of distracters to modulate behaviors, and for pharmaceutical manipulations to mediate the negative aspects of the distracter. Matthews *et al* (2012) showed that anxiety-inducing distracters distractibility can be modulated by antidepressant effects focusing on the blockage of dopamine and norepinephrine reuptake within the mPFC of rats. However, serotonin reuptake has also been implicated in anxiety, and is one of the most common forms of pharmaceutical management of anxiety (Cipriani *et al*, 2009). The serotonin transporter (SERT) has been detected in high density within the cortex of rats (Sur *et al*, 1996), and serotonin's post synaptic terminals are also found in great abundance within the mPFC (Puig *et al*, 2011). Unfortunately, the influence of serotonin upon timing with emotional distracters has not been fully elucidated.

We investigated the role of the prelimbic cortex in the detrimental effect of anxiety-inducing task-irrelevant distracters on the cognitive ability to keep track of time, using local infusions of selective serotonin reuptake inhibitor (SSRI) fluoxetine in a modified peak-interval procedure with neutral and anxiety-inducing distracters. Given that fluoxetine has beneficial effects on attention and working memory, e.g., decreasing emotional response to negative events, we hypothesized that fluoxetine would improve maintenance of information in working memory in trials with distracters, resulting in a decrease of the disruptive effect of emotional events on the timekeeping abilities.

Methods

Subjects. Thirty-three naive 3-4 months old male Sprague-Dawley rats were used. Experimental procedures were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (2011).

Apparatus. We used 12 standard rat operant chambers (MED Associates, St. Albans, VT) housed in sound attenuating cubicles, of which 4 were used for fear conditioning and the other 8 for interval timing. An 85-dB white noise produced by a white-noise generator was first used during fear conditioning, and then later used as an anxiety-inducing distracter during the timing task. The conditioning chambers and the interval timing chambers were made distinctive by uing different box configurations, and different visual, auditory, and odor cues Matthews et al. The fear conditioning chambers contained a dipper entry space for a liquid dipper (not used). Additionally, no levers were inserted in the boxes at any time, and no food was ever given. Pine pellets were placed in the waste pan. In these boxes the grid floor was connected to shockers and scramblers generating a 1-s 0.85-mA foot shock. In contrast, the interval timing chambers contained two levers situated on the front wall of the chamber, of which, only the left lever was used. Forty-five mg precision food pellets were delivered as a reinforcer. The to-be-timed visual stimulus was a 28-V 100-mA house light. A 66-dB background sound produced by a ventilation fan was present throughout the timing session.

Interval timing. After autoshaping, rats received fixed Interval (FI) training sessions, during which the first lever press after 40s was reinforced by food delivery. Afterwards, rats received FI trials randomly intermixed with non-reinforced peak interval (PI) probe trials. Matthews et al

Fear conditioning. Rats were randomly assigned into two groups. FEAR rats received 6 pairings of noise followed by foot shock. NEUTRAL rats were treated identically, but no shock was presented. Rats

received two 30min fear conditioning sessions, one before and one after recovery from surgery, and one fear conditioning session between each testing session.

Surgery. Twenty-six-gauge bilateral cannula guides were implanted in aseptic surgery under isoflurane anesthesia into the prelimbic cortex (AP 2.5mm, ML ±0.6mm, DV -3.5mm). Rats were given at least 6 recovery days before resuming timing and conditioning procedures. Rats received 6 PI re-training sessions before PI+N testing.

Fear test session. After surgery recovery, FEAR rats received 2 presentations of the noise in extinction followed by 2 noise-shock pairings, at 4min intervals. NEUTRAL rats received no shocks during the fear test. Session lasted 20 minutes.

Local infusions. Cannulae injectors aiming at mPFC were lowered into the cannula guides, extending 1mm below the guides. Rats received 0.5µL infusions of either vehicle (n 5% DMSO in saline) or 6µg/side or 0.6µg/side selective serotonin reuptake inhibitor (SSRI) fluoxetine (fluoxetine hydrochloride, Sigma Aldrich, St. Louis, MO), dissolved in vehicle. Infusion order was counterbalanced. See below.

Timing test sessions with noise and drug infusion. Rats received local drug infusions followed by a 3hr session of interval timing testing, during which rats received FI and PI trials randomly intermixed with PI trials with noise (PI+N). PI+N trials were similar to PI trials, except that the 5-s white noise was presented (during to-be-timed stimulus), 5s from the onset of the trial. Infusion sessions were separated by 3 retraining sessions.

Histology. Rats were anesthetized with isoflurane overdose and transcardially perfused with formalin. Brain sections placed on slides, and stained with crystal violet for histological verification (Figure1). Seven rats were eliminated due to incorrect cannula placement, lost cannulae, failure to acquire the timing task. (NEUTRAL n=14, FEAR n=13)

132 === Figure 1 about here ===

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134 *Data Collection and Analysis*. Lever presses during PI and PI+N trials were recorded through a MED
135 Associates interface. Analyses were conducted on a 100s interval-of-interest starting at the onset of the
136 to-be-timed signal for all PI trials, and a 20-120s window for PI+N trials . The average response rate was
137 analyzed as to evaluate: the accuracy of timing (peak time), precision of timing (width of response
138 function), and peak rate of response. A delay in peak timing in PI+N trials relative to PI trials was
139 computed and analyzed statistically (Buhusi and Meck, 2000).

140 We further investigated the effect of the presentation of the noise and the effect of the drug on the
141 dynamics of timing behavior during individual trials. Briefly, during individual trials, the distribution of
142 lever presses can be approximated by a low-high-low function. Analysis algorithms described in
143 (Swearingen and Buhusi, 2010) were used to extract the start and stop times. The start time is the time
144 at which there is a significant increase in response rate during the trial (from the low-to-high states). The
145 stop time is the point during the trial at which there is a significant decrease in response rate (from the
146 high-to-low states). PI Trials without temporal control (about 17% [ARM1]of total trials) were eliminated
147 from individual-trial analyses and there were no exclusion criteria for PI+N trials.

148 Freezing behavior was recorded and scored (in 2.5 s bins) by two independent observers (93.24 ± 1.24%
149 agreement). The percent freezing behavior after the presentation of the noise in extinction was
150 analyzed to evaluate the fear expression indexed by the rate of decay of freezing behavior after the
151 presentation of the noise in extinction. Less fear expression was indexed by more negative decay rates
152 while more fear expression was indexed by more positive decay rates.

153 Timing accuracy (peak time), timing delay after noise, timing precision (width of function), and the start,
154 stop, and median times were submitted to mixed ANOVAs of peak time, with between-subject variable

group (FEAR, NEUTRAL) and within-subject variables trial type (PI, PI+N) and drug (VEH, FLX 0.6ug, FLX 6ug) were followed by Fisher LSD post-hoc comparisons. To further evaluate the relationship between timing delay and fear expression, the slope of regression lines were calculated using t-tests. Analyses were conducted in SPSS v21 (IBM Corp, Armonk, NY) and Statistica v8 (StatSoft, Tulsa, OK). Statistical tests were evaluated at a significance level of 0.05.

Results

No effect of fluoxetine on timing in the absence of distracters. Rats in both groups' timing peaked about the 40s criterion in PI trials, suggesting that fluoxetine has no effect on interval timing in the absence of distracters (Figure 2). These results were supported by a repeated measures mixed ANOVA on PI times with between-subjects variable (NEUTRAL, FEAR) and within-subject variable drug condition (VEH, FLX 0.6ug, FLX 6ug), which failed to indicate a reliable effect of the drug condition on the average peak time, $F(2,50) = 2.29$. These results suggested that fluoxetine has no effect on working memory for time without distraction.

=== Figure 2 about here ===

Fluoxetine decreases distractibility of negative emotional events, but increases distractibility by neutral events. In PI+N trials, fluoxetine infusion decreased the delay relative to PI trials by negative emotional events in FEAR rats, but increased distractibility (large delays) by neutral events in NEUTRAL rats. To evaluate these results we computed a delay in peak time in PI+N trials (with noise) relative to PI trials (without noise), by subtracting the estimated peak time in PI trials from the estimated peak time in PI+N trials (Figure 3). A mixed ANOVA of the time delay with between-subjects variable (NEUTRAL, FEAR) and within-subject variable drug condition (VEH, FLX 0.6ug, FLX 6ug) indicated a main effect of group, $F(1,25)=4.66$, as well as a group x condition interaction, $F(2,50)=9.69$, however failed to find a reliable main effect of drug condition, $F(2,50)=0.72$. These results suggested that fluoxetine appeared to have a

differential effect upon the distractibility of the groups during the working memory for time task when emotional distractions were given.

=== **Figure 3 about here** ===

No effect of fluoxetine on timing precision or response rate. Mixed ANOVAs of timing precision (width of response function) with between-subjects variable (NEUTRAL, FEAR) and within-subject variable drug condition (VEH, FLX 0.6ug, FLX 6ug) failed to indicate significant effects of group, drug, or interactions, all $Fs < 3.09$. These results suggested that the response curves in PI+N sessions were simply delayed from PI sessions, indicative of a delay in responding and not an difference in responding placement.

Individual-trial analyses support fluoxetine effects. Mixed ANOVAs of the start, stop, and median times in PI trials with between-subjects variable (NEUTRAL, FEAR) and within-subject variable drug condition (VEH, FLX 0.6ug, FLX 6ug) failed to indicated any differences during PI trials, all $Fs < 4.0$. These results supported the finding that fluoxetine had no effect in PI trials.

=== **Figure 4 about here** ===

However, significant differences in the start, median, and stop times in the PI+N trials existed supporting the previous results that suggested that the effect of fluoxetine was only seen during distracted trials. Mixed ANOVAs of the start, stop, and median times indicated main effects of group in the start, median and stop times, all $Fs > 5.61$, and a group x drug interaction in the start and median times, $Fs > 3.41$. These results suggest the larger effect of fluoxetine was in the FEAR rats. Also, similarly to what was found in the curve fitting analyses, the range of the individual trial analyses was not significantly different in either PI or PI+N trials, supporting the suggestion that the responding curve was simply delayed.

Connections between timing delay by fear expression. Individual animal data was plotted on a regression line for the mean timing delay and fear expression values. The slope of the regression line was compared to null slope, indicating that there was no relationship. T-tests analyses indicated reliable differences from a null slope for the FEAR group under the vehicle condition, $t(1,12) = 2.53$, but were not significant under the FLX 6ug condition, $t(1,12) = 1.62$. Additionally, the NUETRAL group showed a statistically significant effect under the FLX 6ug condition, $t(1,13) = 2.51$, $p < 0.05$, but were not significant under the vehicle condition, $t(1,13) = 0.77$. These results supported a paradoxical relationship between the drug conditions and the linear regression slopes.

=== Figure 5 about here ===

Discussion

Our results revealed a dissociation of the effects of fluoxetine infusion in prelimbic cortex on timing and working memory for time (resource allocation), and between neutral and anxiety-inducing distraction. Fluoxetine was effective in preventing memory disruption by negative emotional distracters, but in fact increased distractibility by neutral distracters. These results are not due to possible changes of the timing mechanisms, as fluoxetine had no effect on timing in the absence of distracters, and had no effect on the precision of timing or on the response rate. These results were confirmed by curve fitting analyses and by analyses of responses in individual-trials.

Similar to what was found in Heilbronner and Meck (2013), the effect of fluoxetine had no impact on non-interrupted (PI) probe trials. Serotonin is thought to have a modulatory effect on interval timing, working memory and action selection (Ho *et al*, 2002; Rogers, 2011). In the past dopamine has been found to be primarily responsible for general timing and implicated in distractibility during trials that have an interruption (Buhusi, 2003; Buhusi and Meck, 2002), as well as an emotional distraction (Matthews *et al*, 2012). As such it is important to see that fluoxetine has no impact upon timing without

distraction. Our results indicated that increased serotonin signaling within the mPFC had no impact on general timing processes.

Previous studies have also shown a role of SSRI drug dose on anxiolytic effects (Handley and McBlane, 1993; Pinheiro *et al*, 2007; Salchner and Singewald, 2002; Silva *et al*, 1999). [AM2]Similarly, we saw a dose dependent effect in the FEAR group, where the delay was decreased as the fluoxetine dose increased. This result was seen in the timing functions as well as the individual-trial analyses. However, the anxiogenic result seen in the timing functions was not able to be teased apart in the individual-trial analyses. This negative result might be due to a lack of power for the analyses. Previous studies have shown that other analyses have been sensitive enough to find significance in the PI paradigm, in which individual-trial analyses found no significance (Buhusi and Matthews, 2013).

Additionally, the regression lines of the FEAR and NEU groups reversed under the effects of fluoxetine, which supports the paradoxical effect of fluoxetine seen in the timing functions. It is well known that one possible acute side effect of SSRIs is anxiogenesis. There is an existing literature showing paradoxical effects of SSRIs in treatment in both human and animal research (Bagdy *et al*, 2001; Burghardt *et al*, 2004). In human studies, antidepressant drugs have to be administered chronically before therapeutic relief is seen. However, we were able to see an anxiogenic effect by infusing fluoxetine into the mPFC directly, which suggests that the effect of serotonin might not have a role in timing, but in retaining attentional processes within the prefrontal cortex.

Previous research has implicated the prefrontal cortex in decision making (Kim *et al*, 2009), including distraction from memory tasks (Chao and Knight, 1998; Matthews *et al*, 2012). Indeed the prefrontal cortex of rats has been shown to be analogous to the frontal cortex of nonhuman primates and humans (Uylings *et al*, 2003; Vertes, 2004). The prefrontal cortex has also been implicated in working memory for time paradigms, such as the interval timing procedure (Buhusi and Meck, 2005; Kim *et al*, 2009;

Matthews *et al*, 2012; Pessoa, 2008). Previous studies have found a high density of serotonin transporters (SERT) within the cortical areas (Sur *et al*, 1996). SERT is primarily found on the presynaptic dendrites and its role is to remove serotonin from the synapse. However with fluoxetine, the SERT is blocked and serotonin is abnormally available within the synapse.

Animal[AM3] models of PTSD and anxiety disorders have been developed where an animal is given classical conditioning where a stimulus is followed by an inescapable footshock (Brown *et al*, 2007; Matthews *et al*, 2012). Additionally, the time keeping abilities of animals allow scientists to probe working memory. Over time, animals develop an accurate representation of a time criterion which can then be used as a probe to identify brain components in working memory tasks when anxiety provoking situations occur. (Buhusi *et al*, 2005; Matthews *et al*, 2012). During testing sessions, the stimulus was presented as a task-irrelevant distracter during the timing task resulting in a delay in responding suggesting a failure to maintain subjective time in working memory, possibly due to attentional and working memory resources being diverted away from timing, as proposed by the Relative Time-Sharing (RTS) model (Buhusi, 2003; Buhusi *et al*, 2009).

According to the RTS model, both novel and emotional distracters result in attentional processes being reallocated to the processing of an unexpected stimulus. Previously the attentional processes were placed onto the primary timing task, however because attentional processes are limited, attention would be spent on the timing task and upon distracter (Buhusi, 2003; Buhusi *et al*, 2009). However in disorders such as PTSD, greater attentional emphasis is placed onto stressful distracters, taking more of the attentional resources available. The RTS model allows us to hypothesize that the distracter should cause the FEAR group to be shifted in a greater magnitude as compared to the NEU group without any effect of the drug (VEH condition). However, previous studies have shown that the distractibility of a stressful stimulus can be partially eliminated using antidepressants within the mPFC of rats (Matthews *et al*, 2012). The RTS model can explain the paradoxical results. While the distracter is not effective in

delaying the timing functions of the NEU group, the NEU group delayed under the effect of FLX, indicative that increased serotonin redistributed the attentional resources. Conversely, when the distracter was paired (FEAR) and embedded within the timing task the attentional resources were redirected upon the noise processing. However, the addition of serotonin might have caused more resources to be redistributed to processing the timing task. Therefore, we must conclude the serotonin while having no impact on basal timing, is implicated in the reallocating of attentional processes.

In the United States alone, 11% of people over the age of 12 are taking some form of antidepressant medication (Pratt *et al*, 2011), for depression and anxiety disorders. Proper diagnosis of anxiety disorders are critical to produce optimal patient outcomes and misdiagnosis and treatment can exacerbate symptoms. For example, fluoxetine reduces anxiety related symptoms in subjects that demonstrate anxious behavior (Floresco *et al*, 2011). However, in a population that has no acute anxiety, fluoxetine treatment has a paradoxical anxiogenic effect (Gorman *et al*, 1987; Pae *et al*, 2004). Similarly, in our experiment, NEUTRAL rats, which received no noise-shock pairings demonstrated considerable timing delay in PI+N trials under the high dose of FLX, whereas FEAR rats, which received noise-shock pairings, were less delayed by the noise. These results suggest that FLX had a differential effect on the two groups: FLX had an anxiolytic effect when the noise was emotionally charged (FEAR group), but had an anxiogenic effect when the noise was neutral (NEUTRAL group). Moreover, this may also explain the individual differences in the effect of FLX on time delay in our experiment (see Figure 5), as well as individual differences in responsiveness to FLX medication (Oh, 2009) [AM4](Calil, 2001). Fluoxetine is indeed used as an anxiogenic inducer in non-anxious rodents (Bagdy *et al*, 2001), in the same way that *some* patients experience an exacerbation of anxiety under FLX treatment. Together with previous studies, our results support a differential effect of SSRI fluoxetine on working memory for duration related to the context (neutral / fear) in which subjects (patients) are given the treatment. Further studies are required to elucidate the mechanisms of this paradoxical result.

293 Funding and Disclosure

294

295 Acknowledgments

296 This research was supported by the National Institutes of Health through grants MH65561 and
297 MH73057 to CVB. We would like to thank Olivia Hao He and Bogdan Bordeianu for preliminary analyses
298 and J. Daniel Obray and Dan Morrison for excellent assistance with animal training. Author contribution:
299 conceived and designed the experiments: ARM, MB, CVB; performed the experiments: ARM and CDC;
300 analyzed the data: ARM, CDC, BZY, CVB; wrote the paper: ARM, CDC, MB, CVB.

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Figure Legends

Figure 1 Cannulae tip placements. NEUTRAL (n = 14); FEAR (n = 13).

Figure 2 Response functions during PI (solid) and noise interrupted PI+N trials (dashed). Vertical lines indicate estimated peak time in PI (solid) and PI+N trials (dashed). Distance between vertical lines represents the delay after noise presentation (black rectangle). Vehicle (VEH, left), fluoxetine 0.6ug (FLX 0.6ug, center), and fluoxetine (FLX 6ug, right). **p < 0.01. NEUTRAL (n = 14); FEAR (n = 13).

Figure 3 Mean timing delay (± SEM) in PI+N relative to PI trials. Vehicle (VEH, left), fluoxetine 0.6ug (FLX 0.6ug, center), and fluoxetine (FLX 6ug, right). Post-hoc comparisons: * p < 0.05, ** p < 0.01. NEUTRAL (n = 14); FEAR (n = 13).

Figure 4 Mean (± SEM) start (left), median (middle), and stop times (right) in PI+N trials. Post-hoc comparisons: * p < 0.05, ** p < 0.01. NEUTRAL (n = 14); FEAR (n = 13).

Figure 5 Scatterplots of timing delay by fear expression in NEUTRAL (left, n = 14) and FEAR rats (right, n = 13). Vehicle (VEH, dotted lines) and fluoxetine (FLX 6ug, solid lines). Fear expression was indexed by the rate of decay of freezing behavior after the presentation of the noise in extinction. Less fear expression (left side of the x-axis) was indexed by more negative decay rates while more fear expression (right side of the x-axis) was indexed by more positive decay rates.

473 **Figure 1**

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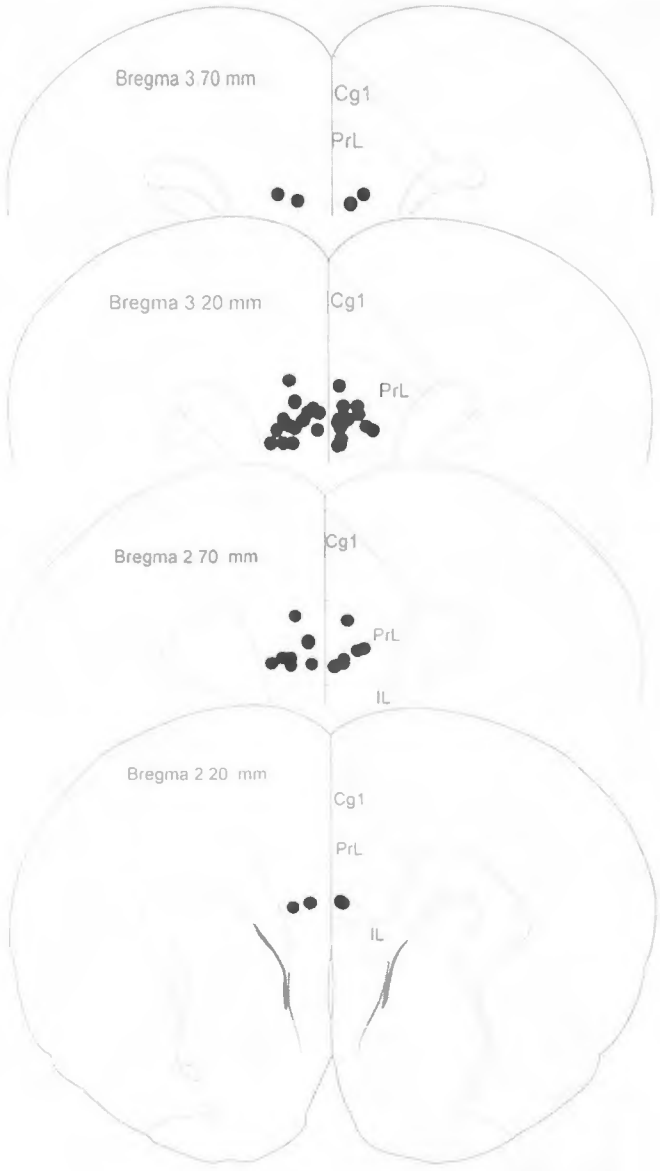

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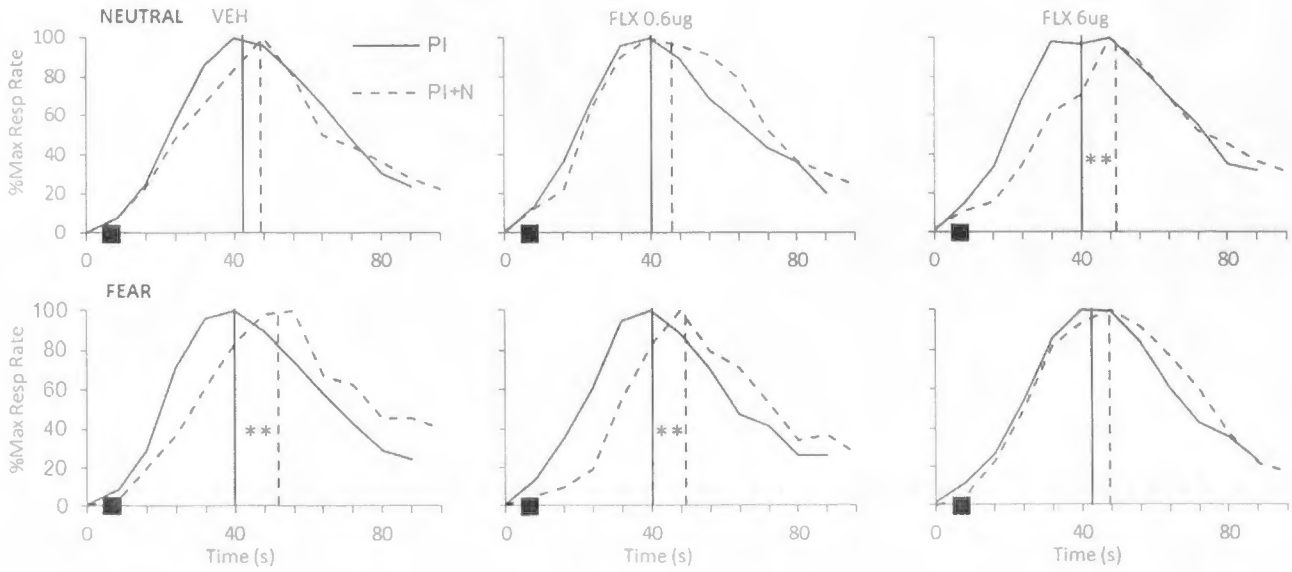
476 **Figure 2**



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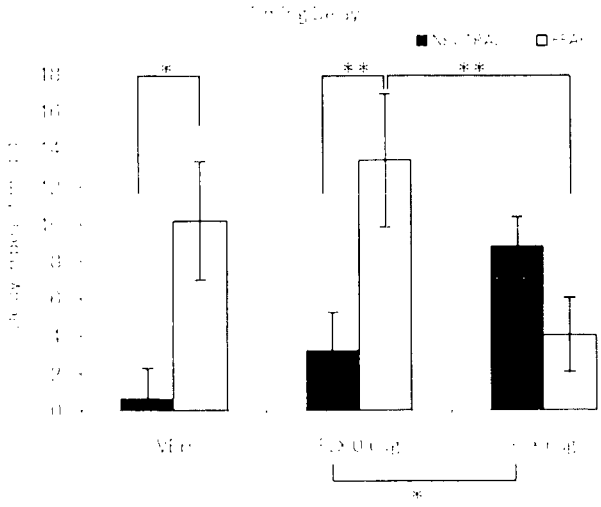
479 **Figure 3**



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483 **Figure 4**

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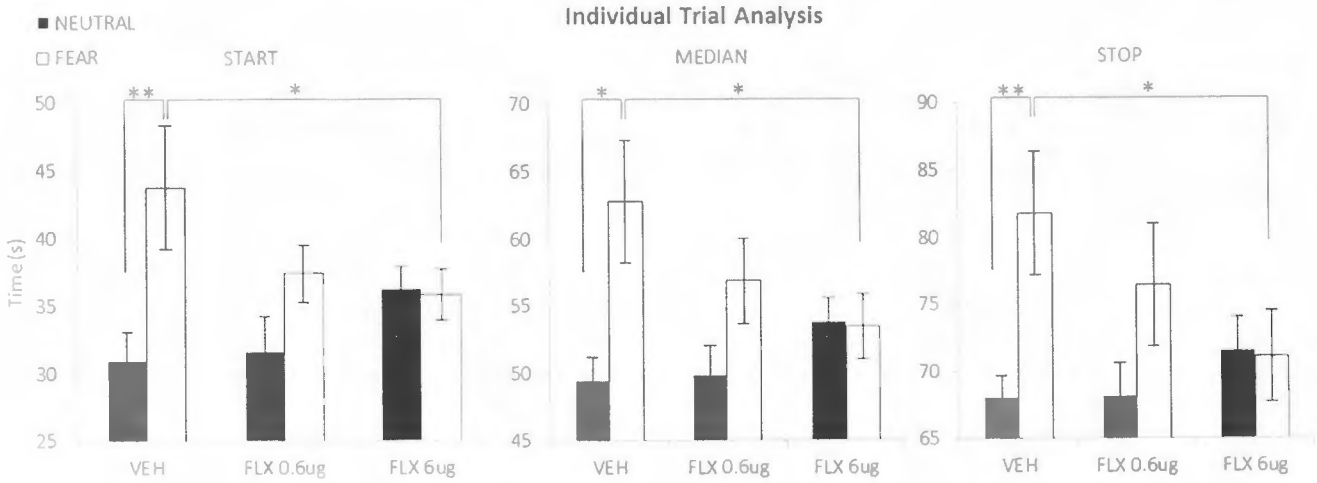

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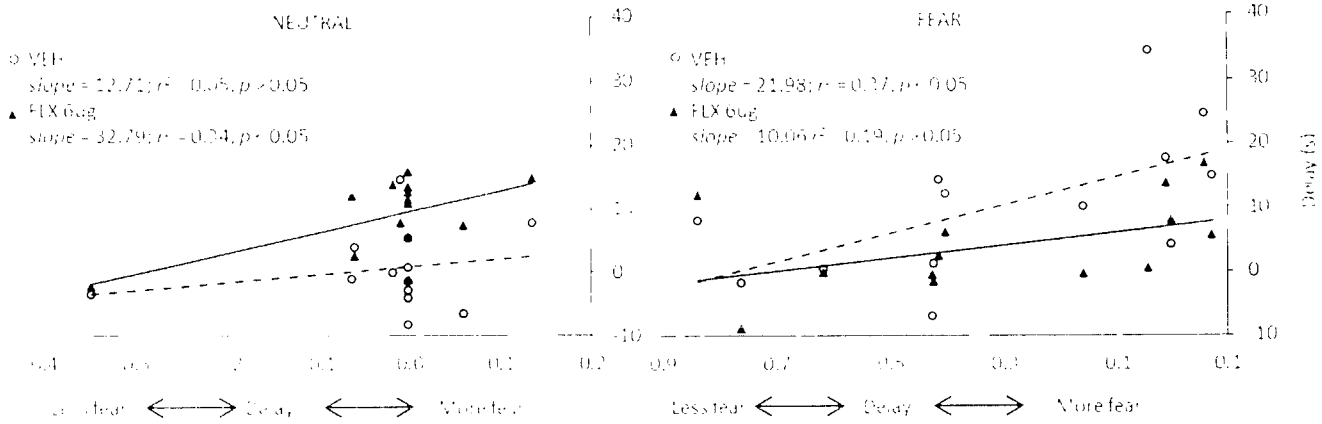
487 **Figure 5**



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